



Patent Application
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Serial No. 10/633,023

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Art Unit : 1656
Applicants : Schnepf, Schwab, Payne, Narva, and Foncerrada
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For : NEMATICIDAL PROTEINS

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

37 CFR §1.132 DECLARATION OF DR. DONALD J. MERLO

Sir:

DR. DONALD J. MERLO hereby declares:

THAT, I received the following degrees:

- 1971-1975: Ph.D. (5/75), Plant Pathology/Biochemistry, University of Wisconsin, Madison, Department of Plant Pathology, Madison, Wisconsin. Studied Molecular Biology of Crown Gall Induction in the laboratory of Dr. John Kemp.
- 1969-1971: M.S. (8/71), Plant Pathology/Botany, University of Nebraska, Department of Plant Pathology, Lincoln, Nebraska. Studied Analysis of Transfer RNA Changes During Fungal Spore Germination in the laboratory of Dr. James Van Etten.
- 1965-1969: B.S. (5/69), Botany, University of Nebraska, Lincoln. Lincoln, Nebraska;

THAT, I have been employed professionally as follows:

Research Scientist, Dow AgroSciences, 1988-present. In the past, have been responsible for supervising molecular biologists focused on discovery and development of transgenic traits for agronomically important crops. Current primary research emphases are development of plant transformation vectors, gene identification and isolation, DNA sequencing, gene expression in microbial and plant systems, and gene design through codon optimization. Also

have been involved in fermentation yield improvement of natural products through microbial strains development. Was co-discoveror of new class of insecticidal proteins produced by *Photorhabdus* and *Xenorhabdus*. Additional personal responsibilities include membership on product development teams, assisting Legal in preparation of patent applications and analysis of patents, and participating in product registration processes.

Research Scientist, Agrigenetics Corporation, Madison, Wisconsin; 1982-1988. Lead the Vector Development group in the construction of plant transformation vectors, gene expression elements, gene cloning and sequencing, and transgenic plant production and analysis. Member of research team that first demonstrated transfer of plant genes across plant species via *Agrobacterium*.

THAT, I have the following additional research experience:

Assistant Professor, University of Missouri, Columbia. Division of Biological Sciences, Columbia, Missouri. (Aug. 1978-Aug. 1982) Research programs included Characterization of plasmids of plant pathogenic *Pseudomonas syringae*, and Cloning of genes encoding the thiamine biosynthesis pathway in *Escherichia coli*. Taught courses in Cell Biology, Molecular Biology Techniques, Plasmids, and Plant Physiology. Was supervisor for Master's Degree and PhD. candidates.

Postdoctoral Research Associate, University of Washington, Seattle, Department of Microbiology and Immunology, Seattle, Washington. (Aug. 1975-Aug. 1978). Studied transformation of plants by *Agrobacterium*. Was member of the research team that first conclusively demonstrated gene transfer from *Agrobacterium* to plants via the Ti plasmid.

THAT, I have been the author or co-author on numerous scientific publications;

THAT, I am an inventor on numerous patents;

THAT I am aware of issues raised in the Office Action dated April 4, 2006;

AND, being thus duly qualified as an expert in the field of the invention who is familiar with the prosecution of the subject patent application, do hereby declare:

If one skilled in the art were to read the subject specification, including Example 5, one would turn to pages 326-328 of the cited Maniatis *et al.* text to determine the hybridization conditions that were used for the hybridization procedures described in Example 5 of the specification.

According to the specification, the subject 52A1 gene (SEQ ID NO:7) was obtained by DNA hybridization procedures as described in Example 5. As described in that Example, recombinant phage containing the 52A1 gene (in a *Sau*3A insert) were plated onto *E. coli* cells, plaques were screened by hybridization to DNA probes, and hybridizing phage were plaque-purified and used to infect liquid cultures of *E. coli* cells for "...isolation of phage DNA by standard procedures (Maniatis *et al.*)."


Maniatis *et al.* is also cited in Example 7 for "isolation of phage DNA by standard procedures", and the full citation of Maniatis *et al.* (1982) is provided in that Example.

Furthermore, with the subject gene (SEQ ID NO:7) and toxin in hand, one skilled in the art could readily use related hybridization probes to screen collections of *Bacillus thuringiensis*, for example, for related genes and toxins.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or of any patent issuing thereon.

Further, declarant sayeth not.


Donald J. Merlo


Date

Attachment: Pages 326-328 of Maniatis (1982)

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HYBRIDIZATION TO NITROCELLULOSE FILTERS CONTAINING REPLICAS OF BACTERIOPHAGE PLAQUES OR BACTERIAL COLONIES

The following protocol is designed for (a) two 20-cm × 30-cm nitrocellulose filters or (b) 30 circular, 82-mm-diameter filters. Appropriate adjustments should be made to the volumes when carrying out hybridization reactions with different numbers or sizes of filters.

1. Float the baked filters on the surface of a tray of 6× SSC until they have become thoroughly wetted from beneath. Submerge the filters for 5 minutes.
2. Transfer the filters (a) to a rectangular, flat-bottomed plastic box (22 cm × 32 cm) or (b) to a circular, glass crystallizing dish. Stack the filters on top of one another.
3. Add (a) 300 ml or (b) 100 ml of prewashing solution. Incubate at 42°C for 1–2 hours.

In this and all subsequent steps, the circular filters in the crystallizing dish should be agitated on a rotating platform so that they do not stick to one another. The large, rectangular filters may be stationary.

The prewashing solution removes from the filters any absorbed medium, fragments of agarose, or loose bacterial debris.

Prewashing solution

50 mM Tris · Cl (pH 8.0)
1 M NaCl
1 mM EDTA
0.1% SDS

4. Pour off the prewashing solution. Incubate the filters for 4–6 hours at 42°C in (a) 100–150 ml or (b) 60 ml of prehybridization solution.

The filters should be completely covered by the prehybridization solution. During prehybridization, sites on the nitrocellulose filter that bind single- or double-stranded DNA nonspecifically become saturated by unlabeled, salmon sperm DNA, SDS, or components in the Denhardt's solution. When using ³²P-labeled cDNA or RNA as a probe, poly(A) should be included in the prehybridization solution and hybridization solutions at a concentration of 1 µg/ml to prevent the probe from binding to T-rich sequences that are found fairly commonly in eukaryotic DNA.

Prehybridization solution

50% formamide
5× Denhardt's solution
5× SSPE
0.1% SDS
100 µg/ml denatured, salmon sperm DNA

After all the components have dissolved, centrifuge the prehybridization solution at 1000g at 15°C for 15 minutes or filter it through Whatman 1MM paper using a Buchner funnel. Sterilize the solution by filtration through disposable Nalgene filters. Store frozen at -20°C in 25-ml aliquots.

Formamide. Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by stirring on a magnetic stirrer with Dowex XG8 mixed-bed resin for 1 hour and filtering twice through Whatman 1MM paper. Deionized formamide should be stored in small aliquots under nitrogen at -70°C.

Denhardt's solution (50×)

Ficoll	5 g
polyvinylpyrrolidone	5 g
BSA (Pentax Fraction V)	5 g
H ₂ O	to 500 ml

20× SSPE. See page 314.

Denatured, salmon sperm DNA. This is prepared as follows: Dissolve the DNA (Sigma Type-III sodium salt) in water at a concentration of 10 mg/ml. If necessary, stir the solution on a magnetic stirrer for 2-4 hours at room temperature to help the DNA to dissolve. Shear the DNA by passing it several times through an 18-gauge hypodermic needle. Boil the DNA for 10 minutes and store at -20°C in small aliquots. Just before use, heat the DNA for 5 minutes in a boiling-water bath. Chill it quickly in ice water.

5. Denature the ³²P-labeled probe DNA by heating for 5 minutes to 100°C. Add the denatured probe to the prehybridization solution covering the filters. Incubate at 42°C until 1-3 × C₀t_{1/2} is achieved (see page 325). During the hybridization, the containers holding the filters should be tightly closed to prevent loss of fluid by evaporation.
6. After the hybridization is completed, discard the hybridization solution. Wash the filters 3-4 times, for 5-10 minutes each wash, in a large volume (300-500 ml) of 2× SSC and 0.1% SDS at room temperature. Invert the filters at least once during washing. At no stage during the washing procedure should the filters be allowed to dry.
7. Wash the filters twice for 1-1.5 hours in (a) 500 ml or (b) 300 ml of a solution of 1× SSC and 0.1% SDS at 68°C. At this point, the background is usually low enough to put the filters on film. If the background is still high or if the experiment demands washing at higher stringencies, immerse the filters for 60 minutes in (a) 500 ml or (b) 300 ml of a solution of 0.2× SSC and 0.1% SDS at 68°C.

8. Dry the filters in air on a sheet of Whatman 3MM paper at room temperature. Tape the filters (numbered side up) onto sheets of 3MM paper and place pieces of tape marked with radioactive ink at several locations on the 3MM paper. These markers serve to align the autoradiograph with the filters.

Radioactive ink is made by mixing a small amount of ^{32}P with a waterproof black ink. We find it convenient to make the ink in three grades: very hot (> 2000 cps on a minimonitor); hot (> 500 cps on a minimonitor); and cool (> 50 cps on a minimonitor). Use a fiber-tipped pen to apply ink of the desired hotness to the pieces of sticky tape.



9. Cover the Whatman 3MM paper and filters in Saran Wrap. Apply to X-ray film (Kodak XR or equivalent) and expose overnight at -70°C with an intensifying screen (see pages 470ff).
10. After development, align the film with the filters using the marks left by the radioactive ink. Use a fiber-tipped pen to mark the film with the position of the asymmetrically located dots on the numbered filters. Tape a piece of tracing paper to the film. Mark the position of positive hybridization signals onto the tracing paper. Also mark (in a different color) the positions of the asymmetrically located dots. Remove the tracing paper from the film. Identify the positive colony or plaque by aligning the dots on the tracing paper with those on the agar plate.

Some batches of nitrocellulose filters swell and distort during hybridization so that it becomes difficult to align the two sets of dots. This problem can be alleviated to some extent by autoclaving the filters before use (see pages 304–305).

11. Each positive plaque should be picked as described in Chapter 2 and placed into 1 ml of SM containing a droplet of chloroform. Often, the alignment of the filters with the plate does not permit identification of a single hybridizing plaque. In this case, an agar plug containing several plaques is picked. An aliquot (usually $50\ \mu\text{l}$ of a 10^{-2} dilution) of the bacteriophages that elute from the agarose plug should be replated so as to obtain approximately 500 plaques on an 85-mm plate. These plaques should then be screened a second time by hybridization. A single, well-isolated, positive plaque should be picked and used to make a plate stock (see page 64).

Each positive bacterial colony should be picked with a sterile toothpick into 2 ml of medium containing the appropriate antibiotics. The bacteria are then replated so as to obtain approximately 300 colonies on an 85-mm plate. If the original colony was picked from an uncrowded area of the original plate, a small number of the secondary colonies should be picked and grown overnight in 2-ml cultures. The plasmids in these bacterial cultures should be isolated and analyzed by one of the methods described in Chapter 11. If the original colony was picked from a very crowded area of the original plate, it may be worthwhile screening the secondary colonies by hybridization before isolating and analyzing plasmid DNA.

—	<u>Cry5Ba1</u>	U19725	Foncerrada & Narva	1997 Bt PS86Q3	
	<u>Cry6Aa1</u>	L07022	Narva et al	1993 Bt PS52A1	
	<u>Cry6Aa2</u>	AF499736	Bai et al	2001 Bt YBT1518	
	<u>Cry6Ba1</u>	L07024	Narva et al	1991 Bt PS69D1	
	<u>Cry7Aa1</u>	M64478	Lambert et al	1992 Bt galleriae PGSI245	
	<u>Cry7Ab1</u>	U04367	Payne & Fu	1994 Bt dakota HD511	
	<u>Cry7Ab2</u>	U04368	Payne & Fu	1994 Bt kumamotoensis 867	
	<u>Cry8Aa1</u>	U04364	Narva & Fu	1992 Bt kumamotoensis	
	<u>Cry8Ba1</u>	U04365	Narva & Fu	1993 Bt kumamotoensis	
	<u>Cry8Bb1</u>	AX543924	Abad et al	2002	
	<u>Cry8Bc1</u>	AX543926	Abad et al	2002	
	<u>Cry8Ca1</u>	U04366	Ogiwara et al.	1995 Bt japonensis Buibui	
	<u>Cry8Ca2</u>	AAR98783	Song et al	2004 Bt HBF-1	
	<u>Cry8Da1</u>	AB089299	Yamamoto & Asano	2002 Bt galleriae	
	<u>Cry8Da2</u>	BD133574	Asano et al	2002 Bt	
	<u>Cry8Da3</u>	BD133575	Asano et al	2002 Bt	
	<u>Cry8Ea1</u>	AY329081	Fuping et al	2003 Bt 185	
	<u>Cry8Fa1</u>	AY551093	Fuping et al	2004 Bt 185	also <u>AAW81032</u>
	<u>Cry8Ga1</u>	AY590188	Fuping et al	2004 Bt HBF-18	
	<u>Cry9Aa1</u>	X58120	Smulevitch et al	1991 Bt galleriae	
	<u>Cry9Aa2</u>	X58534	Gleave et al	1992 Bt DSIR517	
	<u>Cry9Aa like</u>	AAQ52376	Baum et al	2003	incomplete sequence
	<u>Cry9Ba1</u>	X75019	Shevelev et al	1993 Bt galleriae	
	<u>Cry9Bb1</u>	AY758316	Silva-Werneck et al	2004 Bt japonensis	
	<u>Cry9Ca1</u>	Z37527	Lambert et al	1996 Bt tolworthi	
	<u>Cry9Ca2</u>	AAQ52375	Baum et al	2003	
	<u>Cry9Da1</u>	D85560	Asano et al	1997 Bt japonensis N141	
	<u>Cry9Da2</u>	AF042733	Wasano & Ohba	1998 Bt japonensis	
	<u>Cry9Db1</u>	AY971349	Flannagan et al	2005 Bt kurstaki DP1019	
	<u>Cry9Ea1</u>	AB011496	Midoh & Oyama	1998 Bt aizawai SSK-10	
	<u>Cry9Ea2</u>	AF358863	Li et al	2001 Bt B-Hm-16	
	<u>Cry9Eb1</u>	AX189653	Arnaut et al	2001	
	<u>Cry9Ec1</u>	AF093107	Wasano & Ohba	2003 Bt galleriae	
	<u>Cry9Ed1</u>	AY973867	Flannagan et al	2005 Bt kurstaki DP1019	
	<u>Cry9 like</u>	AF093107	Wasano et al	1998 Bt galleriae	insufficient sequence
	<u>Cry10Aa1</u>	M12662	Thorne et al	1986 Bt israelensis	
	<u>Cry10Aa2</u>	E00614	Aran & Toomasu	1996 Bt israelensis ONR-60A	
	<u>Cry10Aa3</u>	AL731825	Berry et al	2002 Bt israelensis	
	<u>Cry11Aa1</u>	M31737	Donovan et al	1988 Bt israelensis	
	<u>Cry11Aa2</u>	M22860	Adams et al	1989 Bt israelensis	
	<u>Cry11Aa3</u>	AL731825	Berry et al	2002 Bt israelensis	
	<u>Cry11Ba1</u>	X86902	Delecluse et al	1995 Bt jegathesan 367	
	<u>Cry11Bb1</u>	AF017416	Orduz et al	1998 Bt medellin	



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Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus; Bacillus cereus group.
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AUTHORS Bai,P., Sun,M. and Yu,Z.
TITLE Nematocidal crystal protein from Bacillus thuringiensis strain YBT-1518
JOURNAL Unpublished
REFERENCE 2 (residues 1 to 475)
AUTHORS Sun,M.
TITLE Direct Submission
JOURNAL Submitted (06-APR-2002) College of Life Science and Technology, Huazhong Agricultural University, Wuhan, Hubei 430070, P. R. China
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